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14. ABSTRACT

We are investigating whether FGF2, a growth factor for many different cell types, merits investigation as a drug development target in a breast cancer therapeutic drug discovery program. We are specifically evaluating whether a "dysinhibition" model of breast cancer progression can justify setting up drug screening initiative to discover agonists. If not, the better approach would be to identify FGF antagonists. To meet this objective we are measuring the natural onset and progression of cancer in a mouse model of spontaneous mammary tumor development. We tumor growth to that in a new line of mice that are cancer prone but that lack one of the presumed regulators of tumor growth, FGF2. Indeed we show that it is involved in cancer progression.

15. SUBJECT TERMS
transgenic mice, growth factors, gene knockout, PyVT mammary cancer

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Fibroblast Growth Factor-2: an Epithelial Ductal Cell Growth Inhibitor that Drops Out in Breast Cancer

Andrew Baird, PhD,
CDMRP-BCRP IDEA Award

1. Introduction

We exploited a genetic mouse model of breast cancer in which mammary tumors spontaneously develop with very high predictability and at very predictable times after birth. The model, called the "PyVT mouse", was created by introducing a cancer-causing gene from polyoma virus (PyV) into the genome of the mouse mammary gland. These mice are otherwise normal except that they all get mammary tumors by 60-85 days of age.

Our idea was to ask two very straightforward questions: (1) what happens to the FGF that is naturally found in the mammary gland when these cancers develop? and (2) what happens to these tumors if there is no FGF in the mammary gland? We reasoned that if FGF is involved in the progression of cancers, then the levels may change as cancer develops. We also reasoned that if there was no FGF present then maybe the natural course of cancer development would change. If it did, then the results would point us to a new target for drug discovery: FGF-dependant breast epithelial cells.

2. Body

The progress is summarized in the SOW table presented below but also highlighted results are presented in the key research accomplishments section. In this funding period, we exploited the genetically modified and backcrossed mouse line, acquire and analyzed data on tumor progression in these genetically modified animals and completed the staining of different markers of tumor growth (FGF/FGFR), angiogenesis (Factor lX) and tumor supproession (Erg4).

Methods.

Mice: Experiments were conducted under the oversight of the Institutional Animal Care and Use Committee of the University of California, San Diego. This project used three strains of mice: (1) wild-type, (2) PyVT mice developing spontaneous mammary tumors, (3) FGF2 deficient mice. Hemizygous PyVT (t/+) males and FGF-/- females were crossed to generate male offspring that are heterozygous for FGF2 and expresses the transgene. PyVT/FGF2+/- males are crossed with female FGF2+/- mice to yield PyVT/FGF2-/- , PyVT/FGF2+/- , and PyVT/FGF2+/+ mice. Genotypes were identified from tail DNA by slot blot analysis using a probe for PyVT and FGF2.

Tumor measurements: We followed cohorts of female PyVT/FGF2-/-, PyVT/FGF2+/-, and PyVT/FGF2-/- mice to evaluate mammary tumor onset, incidence, growth and progression. After weaning, body weights of the mice were recorded weekly and the presence of palpable lesions in the mammary glands were determined. Blinded assessments were done with calipers to measure tumor size in two dimensions. Tumor volumes were calculated using a formula of axb2/2 with a being the length and b the length. Following excessive weight loss or the presence of tumors in excess of 20 mm in length, the mice were killed. Tumor volumes at various time points and tumor weights at necropsy were compared between the three groups using ANOVA followed by a Wilcoxon-Rank test.

Immunohistochemistry: To further characterize mammary tumor development in the absence of FGF2, we performed histological characterization of primary tumors at the early stages of tumor development. Mouse mammary fat pads (MFP) were obtained following euthanasia, perfused with PBS and then fixed with 4% paraformaldehyde (PFA) in PBS, pH 7.4. At the time of immunohistological (IHC) staining, paraffin sections were first deparaffinized in xylene and in progressively more dilute solutions of ethanol. Following this, sections were incubated with Proteinase K (Millipore Cat # 21627 0.2 mg/ml) for 10 minutes. These sections were then blocked in normal goat serum (ABC Rabbit Kit PK-4002) for 1 hour and incubated with either anti-FactorVIII (Biocare) or anti-FGF2, FGFR1, R2, R3 and R4 (Sigma) and Ecrg4 at concentrations of 1:100 to 1:2000 overnight at 4 C. After washing, sections were then incubated with biotin-conjugated secondary antibody for 30 minutes at room temperature. Between each of the following steps, three separate washes were conducted for 3 minutes each. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in

distilled water before the sections were treated with Avidin Biotin Complex (ABC) kit (Vectastatin, Burlingame Ca). For visualization, the sections were incubated with diaminobenzidine substrate for 30 minutes. Following washes, the sections were successively counterstained by incubating in, Hematoxylin, 2% acetic acid, bluing reagent, with separate washes between. Sections were then dehydrated in solutions of progressively more concentrated ethanol and xylene. The cover slips were mounted with Vectamount Mounting Solution. Images were taken with an Olympus FXS100-BSW microscope.

Results

With completion of the breeding program we were able to mine the data collected over the funding period and analyzed parameters beyond the initial markers like appearance, FGF and FGR to include measurements like when did the tumors first arise? did they grow differently in the three lines? Were they distributed at the same sites and were the numbers at each site different. Much of the final data is in the paper currently under review (see appendix A3) and was presented at the Era of Hope meeting in 2011 (Appendix A2) where we were fortunate to get significant feedback from attendees who came to our presentation. These analyses are contained in the summary below.

1. First appearance of tumors: As shown in the example below (Figure 1), the cohort of tumor appearance in different generations of lines could be summarized in graphical form as we measured the tumors every 3-5 days. We examined all animals that were created but only included females in the final analyses. They were differentiated by cage and the presence of a right (RC), left (LC) or no marker cut (NC) on ear. Animals were also followed until they were sacrificed because of tumor size. Occasionally a male PyVT carrier that was being used for breeding purposes would develop a palpable tumor, but this was only observed in 4 instances (one of these positive mice is presented in the figure 1 cohort). Data collected during mammary development showed that FGF KO mice had delayed onset of tumorigenesis and when all were collated (see appendix) they were presented in both the abstract and submitted paper. WT mice began to have palpable tumors by day 65, and by day 80 all mice in the cohort had tumors. In contrast, while some FGF2 KO mice began to have palpable tumors around the same time frame, most FGF2-KO mice showed a significantly delayed progression of tumorigenesis, with some palpable tumors only appearing around day 110. Similarly, FGF2+ het mice showed an intermediate phenotype and the onset of tumorigenesis was earlier than was seen in FGF2 KO mice, but later than was seen in WT. This was important to note because it provided evidence of a gene dosing effect and a decreased expression, rather than only a complete knockout affecting tumor growth.

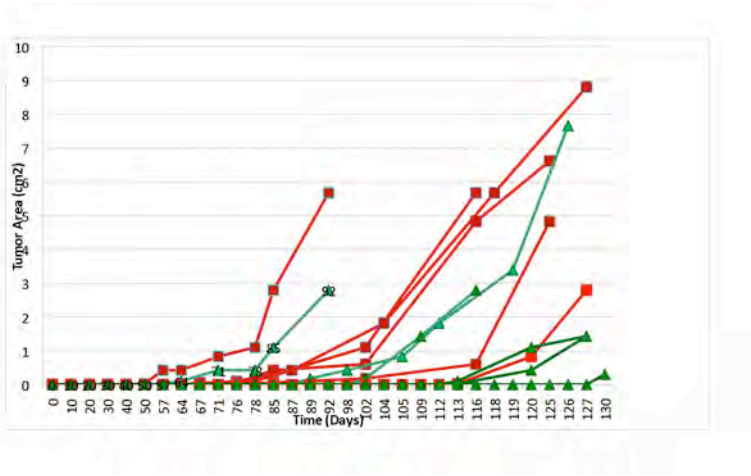

Figure 1: Progression and first appearance of mammary cancers in mouse lines. The distribution of cancer development in the F2 animal cohort is presented here after backcrossing. Each animal was followed to a maximum of 140 days.

2. Tumor size: Other ways of monitoring and comparing tumor growth include measuring onset and tumor mass. Mammary tumors (Figure 2C) in FGF2 KO mice were significantly smaller ($P<0.05$) than those in FGF+ mice (8 vs. 2). This significant difference in tumor burden indicated that FGF2 KO mouse tumor cells grew more slowly and is supported by observation that FGF+ mice were sacrificed at earlier time points (105 vs 134 days). This data is consistent with the data presented earlier indicating delayed onset tumorigenesis. We also noted however that the slope of tumor size increase appeared greater in the FGFKO then in the FGF+ mice. Where the mean time from tumor detection to mouse sacrifice was 20 days in the FGF+ mice, it was 10 days in the FGFKO. It was not because animals were not as healthy but instead because tumors grow faster. This suggests the possibility that there is a secondary FGF-independent pathway that is selected in these animals and that the growth is more aggressive even though delayed.

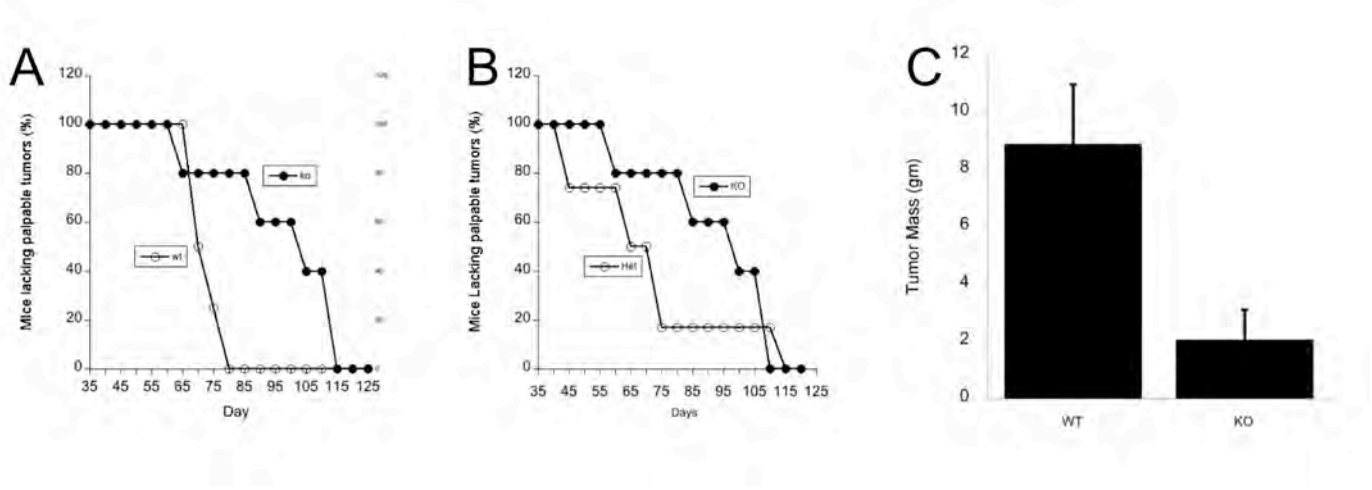

Figure 2: Tumor sizes and growth rates of mammary cancers in the mouse cell lines. The distribution of cancer development in different animal cohort is presented here. Each animal was followed to a maximum of 125 days. In panel A we compared the back crossed line of FGF+ and FGF-KO to detect significant differences that were also found in the comparison of the KO vs Het animals. This suggests a gene dosage effect of FGF.

3. Tumor location: In the course of monitoring tumor development in the mice lines, we also examined the location of tumors and assigned each animal with sites corresponding to different mammary pads (Figure 3A) that included upper (A & D), middle (B & E) and lower (C & F) areas; left and right. We wanted to determine whether the lines showed any preponderance for one location or another for onset but predicted that, because gene expression in the mice lines are not specific, there should be no difference at time of sacrifice. Indeed no differences were observed (Figure 3B) in location when they are compared between groups. The experiment was confounded by the variable incidence of tumors at each site so that in the cumulative analyses of total mass (see paper in Appendix A3) we pooled all data regardless of the site of tumor growth. We also concluded that, as expected, location was not a significant indicator for tumor development particularly when it was compared to the time of appearance or the size of tumors that grow. Like blood vessel number in the tumors, the size of final tumor (Panel C) was different in experimental groups.

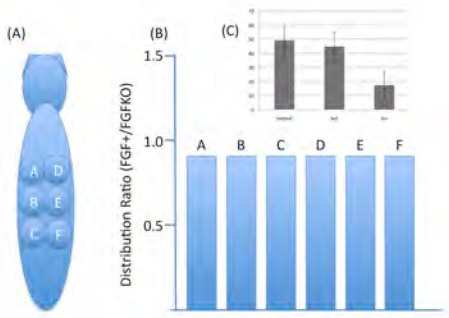

Figure 3: Mammary tumor location at onset and end-stage time points in the mouse lines. The distribution of cancer development in different animal cohorts was evaluated at the time of onset and compared to that at time of sacrifice and given a site of upper, middle of lower left and right.

4. Tumor number at different sites: Another approach for data analyses involved monitoring the number of different tumors at any given site using the same criteria as above (A-F in upper, middle and lower; left and right). Where one tumor would often arise, in fact there were instances in which several palpable tumors would arise at the same site. These analyses proved very difficult because of operator variability and mass fusing over time making it difficult to determine whether one tumor was single or multi-component. While we did an initial analysis of tumor numbers in same loci, we abandoned attempts to quantify individual tumors at each site because end-points were not sufficiently reliable. Differences (Figure 2, Panel C) represent the total tumor mass.

5. Histological appearance: There are several ways to evaluate the tumor state and we had to make a significant number of assumptions in their characterization. For example, the first was that the tumors would be comparable regardless of the site from which they were harvested. To avoid variability however we sought to focus on tumors that developed in the upper-right mammary pad. There were also several endpoints that we used over the funding period and that we discussed in previous reports including myeloperoxidase, collagen lV and Ecrg4. We show here the results with Factor Vlll to visualize blood vessels (Figure 4). In order to quantitatively determine blood vessel density, 3 representative fields each measuring about 1500 µm by 1000 µm on slides stained during the same experiment were analyzed. Blood vessels, as highlighted by IHC for Factor VIII, and confirmed by their morphology, were counted in samples from normal mice, mice lacking one, and mice lacking both copies of FGF2. Average vessel counts from the three groups (each n=3) were then compared. Error bars represent the standard error of the mean (SEM), which was derived from the quotient of the sample standard deviation and the square root of the number of samples. We also performed histological characterization of primary tumors and of mammary fat pads at various stages in development and these results are available in the appendix. Masson's Trichrome (MT) staining in defined ductal structures showed a clear delineation of myoepithelial (MECs) and basal epithelial cells (BECs) separated by basement membrane. Similar ductal structures were seen in tumor-bearing WT mice in regions surrounding the tumor mass as well as in areas of interspersed tumor foci. Thus, the introduction of the PyVT oncogene did not change the gross development of normal epithelial structures in young mice but rather, served to promote the remodeling and progression to tumorigenesis of MECs and BECs. There was a significant difference in the vessel number between the FGF+ and FGF KO mice (Figure 3C)

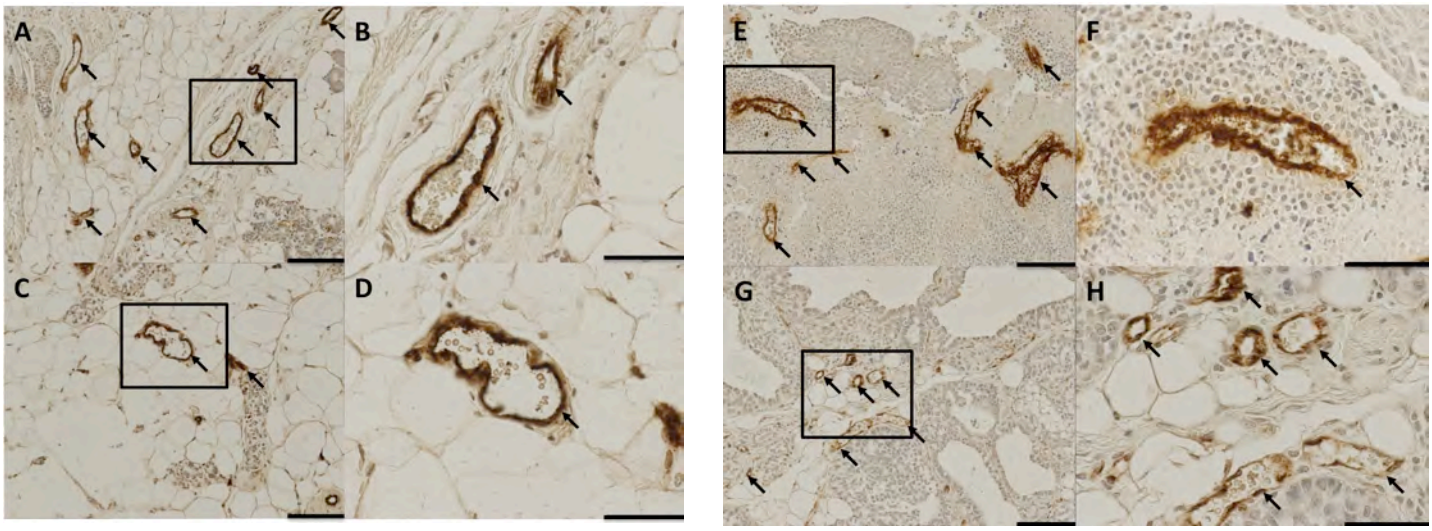

Figure 4: Loss of FGF2 results in decreased tumor vascularization. A: IHC of VWF in a normal lactating mouse mammary fat pad at 17 weeks of age. The inset in A is shown in B. **C:** IHC of VWF in an FGF2 knockout mouse mammary fat pad at 19 weeks of age. The inset in C is shown in D. **E:** IHC of VWF in a PyVT+ mouse mammary fat pad at 22 weeks of age. The inset in E is shown in F. **G:** IHC of VWF in a PyVT+, FGF knockout mouse mammary fat pad at 14 weeks of age. The inset in G is shown in H. Arrows point to positively stained endothelial cells. MEC, myoepithelial cells. BEC, luminal breast epithelial cells. AT, adipose tissue. Scale bars : 100 µm (A, C, E, G); 500 µm (B, D, F, H) . Original magnifications: X400 (A-H)

6. FGF, FGFRs and markers differentiating normal and mammary tumor cells We also continued to characterize the role of FGF2 in normal and tumor-bearing mammary tissue and expanded the description of the FGF receptor. Immunohistochemical staining was performed to determine the presence and localization of FGF2 in breast tumor ductal epithelium. Diffuse staining for FGF2 was present throughout the myo- and basal ductal epithelium of normal (non-PyVT+) (Supplementary Data Figure S1A,B) and transgenic PyVT mammary tumors (Figure S1 C,D) but absent in FGF2 KO (Figure S1 E,F) mice. The similar staining pattern seen in

normal and transgenic PyVT tumors indicated that the introduction of the PyVT oncogene had a limited effect on the expression and localization of endogenous FGF2. In FGF KO tumors, the diffuse staining pattern described earlier was absent, confirming loss of FGF2 immunoreactivity in the knockout. The distribution of FGFR1 (Figure 5) was localized to specific ductal morphological structures and to demonstrate that there exists a potential pathway for FGF2 activity in mammary tissue. Immunohistochemistry staining confirmed the presence of FGFR1 immunoreactivity in both MECs and BECs in normal (Figure 5A,B), PyVT+ and FGF KO (Figure 5E,F) mice. The similar staining patterns seen in the three treatment groups show that FGFR1 expression is not significantly influenced by the presence of the PyVT oncogene or the absence of FGF2.

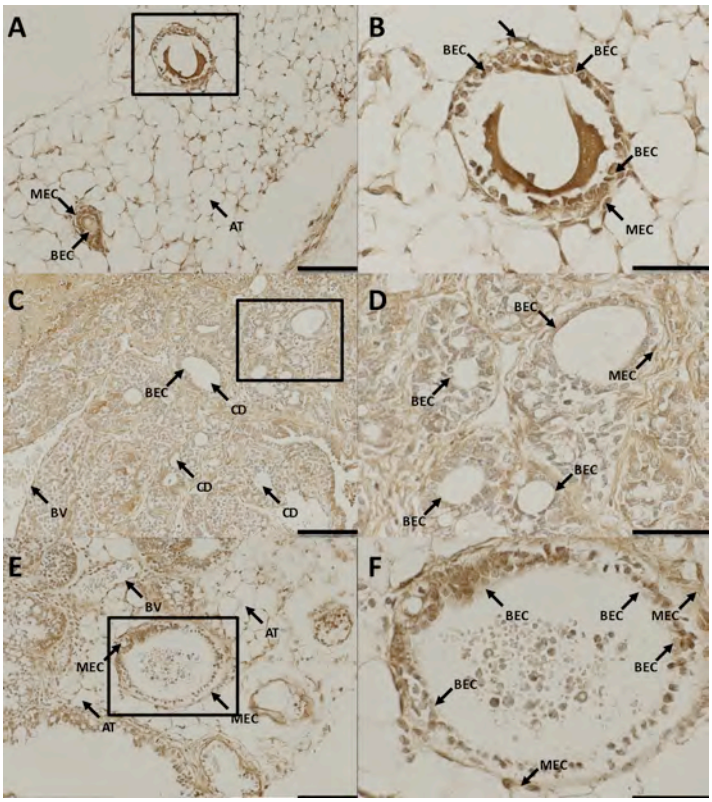

Figure 5 Immunohistochemistry (IHC) of FGFR in mammary cancer. Staining confirmed the presence of FGFR1 immunoreactivity in both MECs and BECs in normal (Panels A & B), PyVT+ and FGF KO (Panels E & F) mice. MEC, myoepithelial cells. BEC, luminal breast epithelial cells

We also evaluated several other markers of mammary tumor progression to determine whether there might be ways to explain the delayed effects of FGF-KO on tumor appearance and progression in the PyVT mice. The marker that we focused on the most was the candidate tumor suppressor gene called Ecrg4 which our preliminary data of the previous year had found was implicated in the development of mammary cancer. The results were mixed and we have been trying to locate better antibodies to confirm the absence of this gene in mammary cancer and potential changes in its expression with changes with FGF.

Task #1 Characterize the distribution of FGF2 and FGFR in mammary wild type mice. (Months 1-12): Complete
Created baseline colony & map normal distribution of FGF and FGR in wt mice

Task #2 Characterize the distribution of FGFR in mammary of FGF2 null mice. (Months 6-12): Complete
Create baseline FGF null colony & map changes in distribution of FGFRs

Task #3 Characterize the distribution of FGFR in mammary of FGF2 over-expressing mice (Months 6-12): Modified and Completed as backcrossed colony.
Animal health precluded creation of baseline FGF2 over-expressing colony to map FGF2 & FGFRs distribution.
Strategy modified to remove animal health variable from tumor growth experiments: back crossing.

Task #4 Characterize the distribution of FGF2 and FGFR in mammary of PyMt mice (Months 6-12): Complete
Created baseline PyMt over-expressing colony & map changes in FGF2 & FGFRs distribution.

Task #5 Characterize the distribution of FGFR in mammary of PyMt x FGF null mice (Months 12-24): Copmplete
Created baseline PyMt$^{+/+}$ FGF$^{-/-}$ colony & map changes in FGF2 & FGFRs.

Task #6 Characterize the distribution of FGF2 and FGFR in mammary of PyMt x FGF over-expressing mice (Months 12-24): Modified and Completed as backcrossed colony.

See task 3 above. Animal health precluded creation of baseline FGF2++ over-expressing colony to map FGF2 & FGFRs distribution. Strategy modified to remove animal health variable from tumor growth experiments: back crossing.

Task #7 Determine kinetics, onset and progression of mammary tumors in wild type mice (Months 1-36):
Complete
Described baseline incidence of spontaneous mammary tumor development in wt/wt mice

Task #8 Determine kinetics, onset and progression of mammary tumors in FGF2 null mice (Months 18-36):
Complete
Baseline described for incidence of spontaneous mammary tumor development in $FGF2^{-/-}$ mice

Task #9 Determine kinetics, onset and progression of mammary tumors FGF2 over-expressing mice (Months 18-36):
Modified and Completed as backcrossed colony.
See task 3 and 6 above. Animal health precluded creation of baseline FGF2++ over-expressing colony.
Strategy modified to remove animal health variable from tumor growth experiments: back crossing.
Proceedurally valid experiments performed with F2 backcrossed and beyond generations.

Task #10 Confirm kinetics, onset and progression of mammary tumors in PyMt mice (Months 12-24):
Complete
Described baseline incidence of spontaneous mammary tumor development in $PyMT^{+/+}$ mice

Task #11 Determine kinetics, onset and progression of mammary tumors in PyMt x FGF-null mice (Months 12-36):
Complete
Described baseline incidence of spontaneous mammary tumor development in $PyMT^{+/+}$ mice

Task #12 Determine kinetics, onset and progression of mammary tumors after PyMt x FGF over-expression (Months 12-36)
Modified and Completed as backcrossed colony.
See task 3, 6 and 9 above. Animal health precluded creation of baseline FGF2++ over-expressing colony.
Strategy modified to remove animal health variable from tumor growth experiments used back crossing.
Proceedurally valid experiments performed with F2 backcrossed and beyond generations.

3. Key Research Accomplishments (bulleted list of important research findings resulting from the achievement of project milestones)

- tumor development is delayed in mice with low endogenous levels of FGF2,
- tumor sizes are smaller in mice with lower FGF2
- Effects of FGF2 are dependant on gene dosage.
- FGF2 is a pro-tumorigenic growth factor.

4. Reportable Outcomes (published or in-press manuscripts, abstracts, presentations, products, patents, grant funding awarded or applied for, and career developments that resulted from this award during the reporting year)

We have presented the findings at the Era of Hope conference in Florida 2012 (see appendix). We have also prepared a first manuscript that we have submitted for publication (se appendix) and are preparing a second describing the unanticipated finding that Ecrg4 is absent in the tumors that arise from these lines (see preliminary results) and suggest that they may be a used as a marker for mammary cancer.

5. Conclusion

These data all point towards the fact that FGF2 is a growth factor in the mouse mammary gland and not a pro-differentiation factor. The new establish that antagonist drug development could result in the identification of new classes of anti-breast cancer drugs. The fact that tumors arise even in the KO mice strongly point to the multiplicity of factors supporting mammary tumor development. Alternatively, the

findings here could also reflect the “transforming” power of the constitutive expression of PyVT in these mice [1-11] and the fact that its effects cannot be readily overcome.

In these studies, we assessed whether FGF2 inhibits the onset, growth and progression of mammary tumors or acts as a pro-angiogenic factor that accelerates tumorigenesis. Specifically, we characterized the expression of FGF2 and FGFR1 in the normal and transgenic mouse mammary glands, and assessed tumor development and progression in FGF2 KO and identified the differences in mammary gland morphology and tumor vascularization. FGF2 and FGFR1 in normal and PyVT-positive mice localized to the myo- and basal epithelium. The data demonstrate that the loss of FGF2 resulted in decreased angiogenesis and a delay in tumor onset. In FGF2 KO mice mammary tumors, blood vessel density was markedly reduced with the loss of FGF2 resulting in delayed tumor growth as well as tumors that were significantly smaller. Importantly, mice that were heterozygous for FGF2 showed an intermediate phenotype in tumor onset and growth. This evidence of a gene dosing effect implies that the action of FGF2 depends on its level of expression rather than an “all-or-none” model of signal transduction. Despite the delay, all of the mice in the cohort eventually developed tumors, suggesting that FGF2 plays at most an ancillary role in tumorigenesis. The results are consistent with the hypothesis that FGF2 contributes to tumor development and the progression to malignancy.

Previous studies implicated FGF2 as a pleiotropic [21] but locally acting cytokine that has distinct juxtacrine roles at the interface been various cell types [15, 22-24]. One of the many established functional roles of endogenous FGF2 is angiogenesis [25], but it is always exogenous FGF2 that has been shown to stimulate endothelial proliferation and tube formation in *in vitro* and *in vivo* models [26, 27]. The role of FGF2 in cancer angiogenesis has been assumed based on its production by most cancer cell lines [28, 29]. However, it is important to note that when breast cancer cell lines are evaluated *in vitro* or in xenograft models to investigate tumor-stroma interactions, the results are often not applicable to *in vivo* tumorigenesis due to differences in the composition of the local extracellular matrix[30-33] and the absence of various tissue specific stromal interactions[34-36]. Furthermore, tumor cells lines are derived from cells at very late stages of tumor progression and selected for growth in cell culture over many passages [37]. At the same time, naturally occurring tumors are rare and unpredictable. In order to overcome these difficulties, we used the MMTV-PyVT mouse model. This model has a high frequency of tumor development [5], where the PyVT is under control of the mouse mammary tumor virus long terminal repeat promoter (MMTV-LTR) [6]. This model has been shown to be an accurate representation of the development of human breast cancer [3, 4]. The studies described here provide the *in vivo* demonstration that loss of FGF2 confers a less malignant phenotype due to reduced vascularization. Furthermore, FGF2 and its receptors have been shown to be highly expressed in many breast cancers[38] and has been shown to have direct mitogenic effects in the mammary epithelium as well as activation of migration [17]. Studies have demonstrated a close interaction between hypoxia and heparin sulfate proteoglycans that modulate the levels of FGF2 signaling [39]. However, previous studies described FGF2 as an upstream mediator of angiogenesis[40] that effects further inflammatory responses that are necessary to complete the process of neo-vascularization [18]. In light of these data as well as the findings presented here, we propose that the action of FGF2—while important in initiating angiogenesis in response to hypoxia-induced stress common in the tumor microenvironment as well as direct mitogenic effects through inhibition of p53, upregulation of survivin [41], and induction of mdm2 [42]—may be superceded by other inflammatory mediators following sufficient inflammatory signaling when it is lost. This hypothesis may harbor significant implications for future therapeutics that attempt to target the action of FGF2. Rather than solely targeting the action of FGF2 on mammary epithelium, there will need to be a combination of therapeutics that will prevent bypassing of FGF2-induced angiogenesis.

There are a variety of clinical studies that have shown that low serum levels of FGF2 correlate with a more malignant phenotype [13], larger tumor size, later disease stage [14], and worse overall and disease-free survival [13, 14]. In addition, many pre-clinical studies have also shown that overexpression of FGF2 inhibits growth [43], although the exact pathway has yet to be elucidated [44]. In contrast, our study shows that the loss of one or both copies of FGF2 results in smaller tumors. Further studies are needed to reconcile this incongruity.

The primary goal of this study was to analyze the contribution of FGF2 to tumor development and progression to malignancy *in vivo* through gene knockout. Because FGF2 KO mice were shown to have delayed tumor development and growth compared to PyVT controls with endogenous levels of FGF2, we suggest that FGF2 promotes tumor angiogenesis and progression to malignancy. This experiment has validated the concept

that FGF2 blockade in the context of breast cancer will be a beneficial therapeutic intervention. The data obtained here demonstrate that the PyVT mammary tumors are sensitive to FGF2 signaling and would identify the PyVT model as an important preclinical model to evaluate therapies aiming to remove FGF2 function. Our results support the conventional view that, presumably because of its powerful mitogenic activity, FGF2 is a pro-tumorigenic growth factor.

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7. Appendices

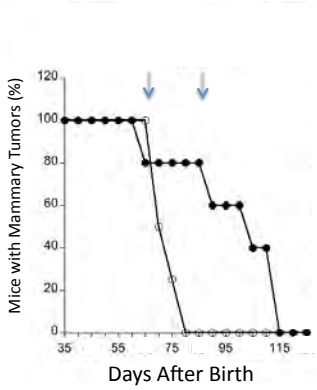
A.1 Abstract submitted to 2011 Era of Hope meeting.
A.2 Poster accepted and then presented at 2011 Era of Hope meeting.
A.3 Paper submitted for publication to BMC cancer.

APPENDIX

A 1: ERA OF HOPE ABSTRACT (SUBMITTED JANUARY 2011)

Although basic fibroblast growth factor (FGF2) was the first pro-angiogenic molecule to be discovered, it has numerous other activities on the growth and differentiation of non-vascular cell types that are both stimulatory and inhibitory, depending on the cell type evaluated, the experimental design used and the context in which it is tested. To this end, we investigated the effects of manipulating endogenous FGF2 on the development of mammary cancer to determine whether its endogenous contribution *in vivo* was pro- or anti-tumorigenic.

Spontaneous mammary tumor progression in the transgenic MMTV-PyVT mouse model ($PyVt^{+/+}$) is a multi-step process that involves all of the cell targets of FGF2 and its presumed roles in angiogenesis and myoepithelium differentiation. Whereas, the myoepithelium is a natural endogenous source of FGF2 in mammary tissue, we hypothesized that an absence of FGF2 might affect the onset and progression of mammary tumor development in $PyVT^{+}$ mice. To this end, we examined the effects of genetically eliminating FGF2 and FGF2 gene dosing in a cross between $PyVT^{+}$ mice and $FGF2^{+/-}$ knockout mice that generate (1) PyVT+:FGF2+/+ (FGF2-WT), (2) PyVT+:$FGF^{+/-}$ (FGF-het) and (3) PyVT+:FGF-/- (FGF2-KO) mice. After backcrossing into common compatible genetic backgrounds, mice were identified by genotyping and blinded assessments of the onset and progression of mammary tumors was performed by palpation and caliper measurments. As expected, female FGF2-WT mice developed mammary tumors starting around 60 days after birth and by 80 days, 100% of FGF2 WT mice had mammary tumors. These values are similar to those published by other investigators for PyVT-induced tumors propagated in other genetic backgrounds. In contrast, 80% of FGF2-KO mice had no palpable tumors until nearly three weeks later (85 days) when 100% of their WT cohort were positive although all FGF-KO mice were tumor bearing at 115 days ($P<.05$). When the onset mammary tumor development and progression curves were compared between FGF-het and FGF-KO mice, there was a small but difference suggesting a gene dosing effect. Initial histological analyses of the tumors in each group suggest that, once progression was initiated, there were few differences in tumor histology.



The significant delay in tumor onset in the FGF2-KO mice vs. FGF2-WT mice is consistent with a functionally permissive role for FGF2 in the kinetics of mammary tumor onset and, while they argue against a central role for FGF2 in mammary cancer progression, they suggest that the function of endogenous myoepithelial-derived FGF2 in the mammary gland is growth stimulatory, rather then differentiation stimulatory.

A- 2 2011 ERA OF HOPE CONFERENCE ON BREAST CANCER, ORLANDO, FLORIDA

P9-5 BASIC FIBROBLAST GROWTH FACTOR IN PYVT-MEDIATED MAMMARY TUMOR PROGRESSION

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ABSTRACT

Although basic fibroblast growth factor (FGF2) was the first pro-angiogenic molecule to be discovered, it has numerous other activities on the growth and differentiation of non-vascular cell types that are both stimulatory and inhibitory, depending on the cell type evaluated, the experimental design used and the context in which it is tested. To this end, we investigated the effects of manipulating endogenous FGF2 on the development of mammary cancer to determine whether its endogenous contribution in vivo was pro-or anti-tumorigenic. Spontaneous mammary tumor progression in the transgenic MMTV-PyVT mouse model (PyVT+/+) is a multi-step process that involves all of the cell targets of FGF2 and its presumed roles in angiogenesis and myoepithelium differentiation. Whereas, the myoepithelium is a natural endogenous source of FGF2 in mammary tissue, we hypothesized that an absence of FGF2 might affect the onset and progression of mammary tumor development in PyVT+ mice. To this end, we examined the effects of genetically eliminating FGF2 and FGF2 gene dosing in a cross between PyVT+ mice and FGF2+/- knockout mice that generate (1) PyVT+:FGF2+/+ (FGF2-WT), (2) PyVT+:FGF+/- (FGF-het) and (3) PyVT +:FGF-/- (FGF2-KO) mice. After backcrossing into common compatible genetic backgrounds, mice were identified by genotyping and blinded assessments of the onset and progression of mammary tumors was performed by palpation and caliper measurements. As expected, female FGF2-WT mice developed mammary tumors starting around 60 days after birth and by 80 days, 100% of FGF2 WT mice had mammary tumors. These values are similar to those published by other investigators for PyVT-induced tumors propagated in other genetic backgrounds. In contrast, 80% of FGF2-KO mice had no palpable tumors until nearly three weeks later (85 days) when 100% of their WT cohort were positive although all FGF-KO mice were tumor bearing at 115 days (P<0.05). When the onset of mammary tumor development and tumor progression curves were compared between FGF-het and FGF-KO mice, there was a small but also significant difference suggesting a gene dosing effect. Initial histological analyses of the tumors suggest that there were few differences in tumor histology. The significant delay in tumor onset in the FGF2-KO mice vs. FGF2-WT mice was consistent with a functional role for FGF2 in mammary tumor onset but argue against a central role for FGF2 in mammary cancer progression via targeted differentiation.

INTRODUCTION

Breast tissue has been shown to have extraordinary plasticity and a high level of interaction between breast myoepithelial cells (MEC) and luminal breast epithelial cells (BEC). This interaction is thought to be important in the development and function of normal mammary gland[1,2]. The loss of inhibitory processes[3,4] could lead to positive feedback loops that can allow for tumor progression. We studied the effect of the loss of fibroblast growth factor-2 (FGF2), a myoepithelial protein that is conventionally seen as a proliferation factor, to determine whether it may exert an effect on tumorigenesis through BEC growth inhibition. Endogenous FGF2 controls a series of functions at cell-cell interfaces between MECs and BECs. Namely, FGF2 is expressed by MECs and localizes at their interface with luminal BECs to act as a survival and differentiation factor. In breast cancer, MECs are lost along with their secreted FGF2. Clinically, low levels of FGF2 in breast cancer are associated with a poor prognosis and response to treatment.

Breast cancer progression may thus be driven by dysinhibition of BEC that results when FGF2 expression at the MEC-BEC interface is lost. This study evaluates the role of endogenous FGF2 expression on mammary tumor development in the PyMT transgenic mouse mammary tumor model in which mammary tumors are driven by the polyoma middle T oncogene. Since FGF2 exerts competing effects on tumor progression—through inhibition at the MEC-BEC interface, as well as acceleration due to its angiogenic effects—this study will attempt to elucidate which of the effects of FGF2 is predominant in tumorigenesis.

METHODS

Mice: This project used three strains of mice: (1) wild-type, (2) PyMT mice developing spontaneous mammary tumors, and (3) FGF2 deficient mice. Hemizygous PyMT (t/+) males and FGF-/- females were crossed to generate male offspring that are heterozygous for FGF2 and expresses the transgene. PyMT/FGF2+/- males were crossed with female FGF2+/- mice to yield PyMT/FGF2-/- , PyMT/FGF2+/- , and PyMT/FGF2+/+ mice.

Tumor measurements: We followed cohorts of female PyMT/FGF2-/-, PyMT/FGF2+/-, and PyMT/FGF2-/- mice to evaluate mammary tumor onset, incidence, growth and progression. After weaning, body weights of the mice were recorded weekly and the presence of palpable lesions in the mammary glands were determined. Blinded assessments were done with calipers to measure tumor size in two dimensions. Following excessive weight loss or the presence of tumors in excess of 20 mm in length, the mice were killed. Tumor volumes at various time points and tumor weights at necropsy were compared between the three groups using a Wilcoxon-Rank test.

Immunohistochemistry: To further characterize mammary tumor development in the absence of FGF2, we performed histological characterization of primary tumors at the early stages of tumor development. Mouse mammary fat pads (MFP) were obtained following euthanasia, perfused with PBS and then fixed with 4% paraformaldehyde (PFA) in PBS, pH 7.4. Paraffin sections were first deparaffinized in xylene and in progressively more dilute solutions of alcohol. Following this, sections were incubated with Proteinase K (Millipore Cat # 21627 0.2 mg/ml) for 10 minutes. These sections were then blocked in normal goat serum (ABC Rabbit Kit PK-4002) for 1 hour and incubated with either anti-FactorVIII (Biocare) or anti-FGF2 and anti-FGFR1 (Sigma) at concentrations of 1:100, 1:4000 and 1:1000 respectively overnight at 4 C. Sections were then incubated with biotin-conjugated secondary antibody for 30 minutes at room temperature. Following endogenous peroxidase activity quenching, the sections were treated with Avidin-Biotin Complex (ABC) kit (Vectastatin, Burlingame Ca). For visualization, the sections were incubated with diaminobenzidine substrate (DAB).

LOSS OF FGF2 DELAYS TUMOR PROGRESSION AND INHIBITS TUMOR GROWTH

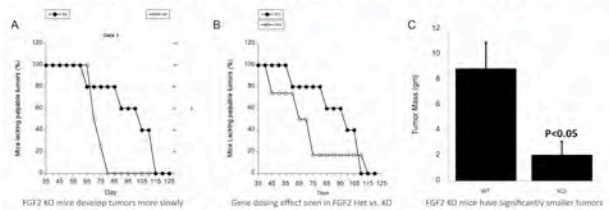

Figure 1. Delayed progression of tumorigenesis and decreased tumor size in knockout and heterozygous mice for FGF2 as compared to normal mice. A: Tumorigenesis in knockout and wild-type mice. **B:** Tumorigenesis in knockout and heterozygous mice. Data collected during mammary development show that mice lacking the FGF2 gene have delayed onset of tumorigenesis. Wild-type mice begin to have palpable tumors by day 65 and by day 80, all have tumors. Some FGF2 KO mice begin to have palpable tumors around the same time. However, most knockout mice show a delayed progression of tumorigenesis, with some only beginning to have palpable tumors around day 110. Similarly, mice heterozygous for FGF2 show an intermediate phenotype in that the onset of tumorigenesis is earlier than was seen in FGF2 KO mice, but later than was seen in FGF2 WT. **C:** Mammary tumors in FGF2 knockout mice are significantly smaller than those in normal mice. Tumors in normal mice average 8 grams as compared to 2 grams in mice lacking FGF2 at time of necropsy. (P<0.05)

FGF2 and FGFR1 EXPRESSION IN MAMMARY TUMORS

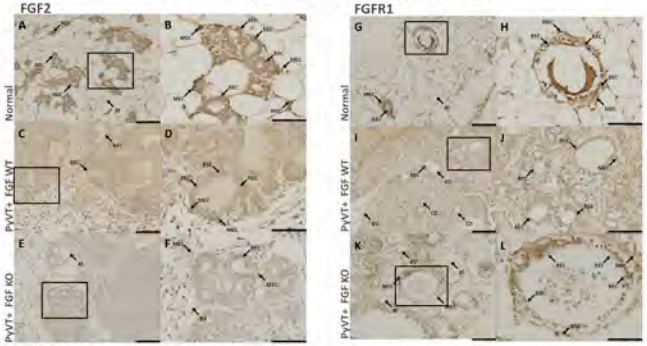

Figure 4. FGF2 in PyVT+ localizes to MECs while FGFR1s localize to BECs. A: IHC of FGF2 in a normal mouse mammary fat pad at 17 weeks of age. The inset in A is shown in B. **C:** IHC of FGF2 in a PyVT+ mouse mammary fat pad. The inset in C is shown in D. **E:** IHC of FGF2 in a PyVT+, FGF2 KO mouse mammary tumor at 19 weeks of age. The inset in E is shown in F. **G:** IHC of FGFR1 in a PyVT+, FGF2 KO mouse mammary tumor at 10 weeks of age. The inset in G is shown in H. **I:** IHC of FGFR1 in a PyVT+ mouse mammary tumor at 22 weeks of age. The inset in I is shown in J. **K:** IHC of FGFR1 in a PyVT+, FGF2 KO mouse mammary tumor at 14 weeks of age. The inset in K is shown in L. MEC, myoepithelial cells. BEC, luminal breast epithelial cells. BV, blood vessel. AT, adipose tissue. CD, collecting ducts. Scale bars: 100 µm (A, C, E, G, I, K). 50 µm (B, D, F, H, J, L) . Original magnifications: X400 (A-L)

DUCTAL HISTOLOGY IN NORMAL AND PyVT+ FGF2 WT AND KO

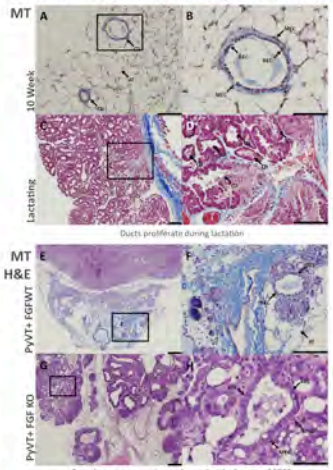

Figure 2. Ductal morphology in PyVT- is comparable to PyVT+. A: Masson's Trichromic staining of mammary gland from normal mouse at 10 weeks of age. The inset in A is shown in B. **C:** Masson's Trichrome staining of mammary gland prepared from a normal lactating mouse at 26 weeks of age. The inset in C is shown in D. CD, collecting milk ducts. **E:** Masson's Trichrome staining of mammary gland from PyVT+ mouse at 22 weeks of age. The inset in E is shown in F. **G:** H&E staining of mammary gland from PyVT+, FGF KO mouse at 14 weeks of age. The inset in G is shown in H. CD, collecting milk ducts. MEC, myoepithelial cells. BEC, luminal breast epithelial cells. AT, adipose tissue. Scale bars: 200 µm (C, E, G); 100 µm (A, D, F, H); 50 µm (B). Original magnifications: X200 (C, E, G); X400 (A, D, F, H); X600 (B)

VESSEL DENSITY & MORPHOLOGY IN NORMAL AND FGF2 KO

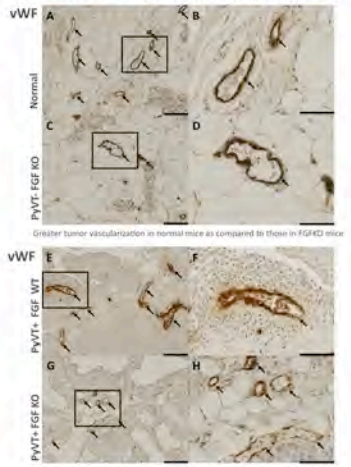

Figure 3. Loss of FGF2 results in decreased tumor vascularization. A: IHC of VWF in a normal lactating mouse mammary fat pad at 17 weeks of age. The inset in A is shown in B. **C:** IHC of VWF in an FGF2 knockout mouse mammary fat pad at 19 weeks of age. The inset in C is shown in D. **E:** IHC of VWF in a PyVT+ mouse mammary fat pad at 22 weeks of age. The inset in E is shown in F. **G:** IHC of VWF in a PyVT+, FGF knockout mouse mammary fat pad at 14 weeks of age. The inset in G is shown in H. Arrows point to positively stained endothelial cells. MEC, myoepithelial cells. BEC, luminal breast epithelial cells. AT, adipose tissue. Scale bars : 100 µm (A, C, E, G); 500 µm (B, D, F, H) . Original magnifications: X400 (A-H)

CONCLUSIONS

The primary goal of this study was to analyze the contribution of FGF2 to tumor development and progression. Since FGF2-/- mice were shown to have delayed tumor development and growth compared to PyMT controls with endogenous levels of FGF2, we showed that FGF2 promotes tumor angiogenesis and progression to malignancy. This experiment has validated the idea that removing FGF2 in the context of breast cancer will be a beneficial therapeutic intervention. The data obtained here demonstrate that the PyMT mammary tumors are sensitive to FGF2 signaling and would identify the PyMT model as an important preclinical model to evaluate therapies aiming to remove FGF2 function. From the data presented, it is evident that no dysinhibition from FGF2 may be observed and tumor growth slows with FGF2 loss. This confirms the conventional view that, because of its powerful angiogenic activity, FGF2 is a pro-tumorigenic growth factor.

Funded by a CDMRP-IDEA Research Grant (W81XWH-08-1-0708)

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A-3 Manuscript (submitted)

Basic Fibroblast Growth Factor In An Animal Model Of Spontaneous Mammary Tumor Progression

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ABSTRACT

Although basic fibroblast growth factor (FGF2) was the first pro-angiogenic molecule discovered, it has numerous activities on the growth and differentiation of non-vascular cell types. FGF2 is both stimulatory and inhibitory, depending on the cell type evaluated, the experimental design used and the context in which it is tested. Here we investigated the effects of manipulating endogenous FGF2 on the development of mammary cancer to determine whether its endogenous contribution in vivo was pro- or anti-tumorigenic. Specifically, we examined the effects of FGF2 gene dosing in a cross between a spontaneous breast tumor model (PyVT+ mice) and $FGF2^{-/-}$ (FGF KO) mice. Using these mice, the onset and progression of mammary tumors was determined. As predicted, female FGF2 WT mice developed mammary tumors starting around 60 days after birth and by 80 days, 100% of FGF2 WT female mice had mammary tumors. In contrast, 80% of FGF2 KO female mice had no palpable tumors until nearly three weeks later (85 days) at times when 100% of the WT cohort was tumor positive. All FGF KO mice were tumor-bearing by 115 days. When we compared the onset of mammary tumor development and the tumor progression curves between FGF het and FGF KO mice, we observed a difference, which suggested a gene dosing effect. Analysis of the tumors demonstrated that there were significant differences in tumor size depending on FGF2 status. The delay in tumor onset supports a functional role for FGF2 in mammary tumor progression, but argue against an essential role for FGF2 in overall mammary tumor progression.

INTRODUCTION

The prevalence of breast cancer remains a significant clinical problem, where xenograft models using established tumor cell lines have limitations for the study of mechanisms regulating tumor onset [1]. In contrast, transgenic models such as the polyomavirus middle-T oncogene (PyVT) mouse model driven by the mouse mammary tumor virus (MMTV) promoter, have been shown to be a reliable and comparable mouse model of breast tumor onset and progression [2-4]. The steps of spontaneous mouse mammary tumor progression in this model parallel the histological progression in human breast cancer [3, 5-7].

Breast and mammary tissues have been shown to have extraordinary plasticity during adult life and are thought to depend, at least in part on inhibitory interactions between mammary myoepithelial cells (MECs) and luminal breast epithelial cells (BECs) [8]. Indeed, these same interactions are thought to be important in normal development and function of the mammary gland [8, 9]. Several years ago, investigators proposed that a loss of inhibitory processes [2, 10] might disinhibit positive feedback loops thereby allowing for tumor progression [11]. One candidate is FGF2, which controls epithelial function at the MEC and BEC interface and acts as a survival and differentiation factor[12]. While low levels of FGF2 in breast cancer are associated with a poor prognosis and response to treatment[13, 14] there is evidence that FGF2 overexpression results in enhanced tumor growth due to increased angiogenesis[15]. This discrepancy may highlight that FGF2 signaling can potentially have a dual role of suppressing and promoting tumor growth.

We investigated the possibility that breast cancer progression may be driven in part by a disinhibition of BEC that results when FGF2 expression at the MEC-BEC interface is lost. The progression of spontaneous mammary tumors in the transgenic MMTV-PyVT mouse model ($PyVT^{+/+}$) is a multi-step process[2] that involves many of the cell targets of FGF2[5, 16-18]. Because the myoepithelium is a natural endogenous source of FGF2 in mammary tissue[12], we hypothesized that an absence of FGF2 might affect the onset and progression of mammary tumor development in PyVT+ mice. In the event that endogenous FGF2 might exert competing effects on tumor progression—through inhibition of epithelial cells at the MEC-BEC interface, or alternatively due to its angiogenic effects, we evaluated its activity in a model of spontaneous tumor development.

MATERIALS AND METHODS

Mice: All animal studies were conducted with the approval of, and under the oversight of the Institutional Animal Care and Use Committee of the University of California, San Diego. We bred $FGF^{-/-}$ SV129/Black Swiss female mice with transgenic hemizygous MMTV-PyVT FVB male mice (PyVT+) to yield $FGF2^{-/-}$; PyVT+ (FGF2 KO), $FGF^{+/-}$;PyVT+ (FGF2 het), and $FGF2^{+/+}$; PyVT+ (WT). Tissue expression studies were conducted using tissue harvested from mice that were collected after cervical dislocation. Genotypes were identified from tail DNA by PCR using PCR primers specific for PyVT and FGF2.

Tumor measurements: Cohorts of female WT, FGF2 het, and FGF2 KO mice were followed to evaluate mammary tumor onset, incidence, growth and progression. After weaning, body weights of the mice were recorded weekly and the presence of palpable lesions in the mammary glands was determined. Following excessive weight loss or the presence of tumors in excess of 15 mm in width or length from caliper measurements, the mice were killed. Tumor volumes at various time points and tumor weights at necropsy were compared between the three groups using a Wilcoxon-Rank test.

Antibodies: To demonstrate FGF2 immunoreactivity, a polyclonal antibody was raised in rabbits by immunization against peptides 1-24 of bovine FGF2. This antibody has high affinity for extracellular FGF2 and cross-reactivity with FGF2 from several species, as described previously[19, 20]. Antibodies for Factor VIII and FGFR1 were obtained from BioCare Inc. (Concord, CA, USA) and Sigma Aldrich (St. Louis, MO, USA), respectively.

Immunohistochemistry: Mouse mammary fat pads (MFP) were obtained following euthanasia of animals, perfusion with PBS and fixation with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4. Formalin fixed lung specimens were stored in paraffin by UCSD Histology Core Services. At the time of immunohistological staining, paraffin sections were first deparaffinized in xylene and in progressively more dilute aqueous solutions of ethanol. Sections were then incubated with Proteinase K (Millipore Cat #21627 0.2 mg/ml) for 10 minutes at room temperature. Sections were blocked with normal goat serum (ABC Rabbit Kit PK-4002) in PBS for 1 hour and incubated with primary antibody for Factor VIII (Biocare Inc., Concord, CA, USA) antibody or FGF2 and FGFR1 (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 1:100, 1:4000, and 1:2000 overnight at 4°C in 1% bovine serum albumin in PBS. Sections were washed and incubated with biotin-conjugated secondary antibody (ABC Rabbit Kit PK-4002) for 30 minutes at room temperature. Sections were washed again and incubated in 0.3% H_2O_2 to quench endogenous peroxidase activity for 20 minutes before the sections were treated with an

Avidin Biotin Complex (ABC) (Vectastatin, Burlingame, CA) for amplification of signal. Sections were incubated with diaminobenzidine substrate for 15 minutes and after washing, were counterstained with hematoxylin and dehydrated. The coverslips were mounted with Vectamount Mounting Solution and imaged with an Olympus FXS100-BSW microscope.

Quantification of Vessel Density: In order to quantitatively determine the effect of FGF knockout on blood vessel density, 3 representative fields each measuring about 1500 µm by 1000 µm on slides stained during the same experiment were analyzed. Blood vessels, as highlighted by IHC for Factor VIII, and confirmed by their morphology, were counted in samples from normal mice, mice lacking one, and mice lacking both copies of FGF2. Average vessel counts from the three groups (each n=3) were then compared. Error bars represent the standard error of the mean (SEM).

RESULTS

Delayed progression of tumorigenesis and decreased tumor size in FGF2 knockout and heterozygous mice. The development of tumors in normal mice (WT) and mice lacking one (FGF2 het) or both copies of FGF2 (FGF2 KO) (Figure 1A, B) was marked by significant differences in kinetics. Data collected during mammary development showed that FGF KO mice had delayed onset of tumorigenesis. WT mice began to have palpable tumors by day 65, and by day 80 all mice in the cohort had tumors. In contrast, while some FGF2 KO mice began to have palpable tumors around the same time frame, most FGF2-KO mice showed a significantly delayed progression of tumorigenesis, with some palpable tumors only appearing around day 110. Similarly, FGF2 het mice showed an intermediate phenotype and the onset of tumorigenesis was earlier than was seen in FGF2 KO mice, but later than was seen in WT. This was important to note because it provided evidence of a gene dosing effect and a decreased expression, rather than only a complete knockout affecting tumor growth. Mammary tumors (Figure 1C) in FGF2 KO mice were also significantly smaller ($P<0.05$) than those in WT mice (8 grams in WT mice vs. 2 grams in, FGF2 KO mice). The significant difference in tumor burden indicated that FGF2 KO mouse tumor cells grew more slowly and/or die at a higher rate than those in WT mice tumors and was consistent with the data presented earlier indicating delayed onset tumorigenesis.

Ductal morphology of normal mice mammary gland is comparable to that observed in PyVT+ mice. To further characterize mammary tumor development in the absence of FGF2, we performed histological characterization of primary tumors and of mammary fat pads at various stages in development. Masson's Trichrome (MT) staining of mammary gland highlighted ductal morphology in normal mouse at 10 (Figure 2A,B) and 26 (Figure 2C,D) weeks of age. MT staining in defined ductal structures showed a clear delineation of myoepithelial (MECs) and basal epithelial cells (BECs) separated by basement membrane. Similar ductal structures (Figure 2E-H) were seen in tumor-bearing WT mice in regions surrounding the tumor mass (Figure 2E) as well as in areas of interspersed tumor foci (Figure 2G). Thus, the introduction of the PyVT oncogene did not change the gross development of normal epithelial structures in young mice but rather, served to promote the remodeling and progression to tumorigenesis of MECs and BECs.

Loss of FGF2 results in decreased MFP vascularization in both normal and PyVT+ mice. Immunohistochemistry staining for Factor VIII highlighted the tumor vasculature and enabled quantification of tumor angiogenesis (Figure 3). Normal mice (Figure 3A,B) demonstrated greater vascular density compared to FGF2 KO mice (Figure 3C,D; arrows) at 17-19 weeks of development.

Similarly, tumor-bearing WT mice (Figure 3E,F; arrows) also showed greater vascular density as compared to FGF2 KO (Figure 3G,H; arrows). Furthermore, blood vessel density was significantly lower (Figure 3I) in FGF KO mice mammary tumors when compared to FGF-WT, demonstrating a role for FGF2 in mammary tumor angiogenesis.

FGF2 in mammary tissue of normal, WT, and FGF2-KO mice. To characterize the role of FGF2 in normal and tumor-bearing mammary tissue, immunohistochemical staining was performed to determine the presence and localization of FGF2 in breast tumor ductal epithelium. Diffuse staining for FGF2 was present throughout the myo- and basal ductal epithelium of normal (non-PyVT+) (Figure 4A,B) and transgenic PyVT mammary tumors (Figure 4C,D) but absent in FGF2 KO (Figure 4E,F) mice. The similar staining pattern seen in normal and transgenic PyVT tumors indicated that the introduction of the PyVT oncogene had a limited effect on the expression and localization of endogenous FGF2. In FGF KO tumors, the diffuse staining pattern described earlier was absent, confirming loss of FGF2 immunoreactivity in the knockout.

FGF receptor type 1 (FGFR1) in mammary tissue of normal, WT, and FGF2-KO mice. The distribution of FGFR1 was localized to specific ductal morphological structures and demonstrated that there exists a potential pathway for FGF2 activity in mammary tissue. Immunohistochemistry staining confirmed the presence of FGFR1 immunoreactivity in both MECs and BECs in normal (Figure 5A,B), PyVT+ and FGF KO (Figure 5E,F) mice. The similar staining patterns seen in the three treatment groups show that FGFR1 expression is not significantly influenced by the presence of the PyVT oncogene or the absence of FGF2.

DISCUSSION

In this study, we assessed whether FGF2 inhibits the onset, growth and progression of mammary tumors or acts as a pro-angiogenic factor that accelerates tumorigenesis. Specifically, we characterized the expression of FGF2 and FGFR1 in the normal and transgenic mouse mammary glands, and assessed tumor development and progression in FGF2 KO and identified the differences in mammary gland morphology and tumor vascularization. FGF2 and FGFR1 in normal and PyVT-positive mice localized to the myo- and basal epithelium. The data demonstrate that the loss of FGF2 resulted in decreased angiogenesis and a delay in tumor onset. In FGF2 KO mice mammary tumors, blood vessel density was markedly reduced with the loss of FGF2 resulting in delayed tumor growth as well as tumors that were significantly smaller. Importantly, mice that were heterozygous for FGF2 showed an intermediate phenotype in tumor onset and growth. This evidence of a gene dosing effect implies that the action of FGF2 depends on its level of expression rather than an "all-or-none" model of signal transduction. Despite the delay, all of the mice in the cohort eventually developed tumors, suggesting that FGF2 plays at most an ancillary role in tumorigenesis. The results are consistent with the hypothesis that FGF2 contributes to tumor development and the progression to malignancy.

Previous studies implicated FGF2 as a pleiotropic [21] but locally acting cytokine that has distinct juxtacrine roles at the interface been various cell types [15, 22-24]. One of the many established functional roles of endogenous FGF2 is angiogenesis [25], but it is always exogenous FGF2 that has been shown to stimulate endothelial proliferation and tube formation in *in vitro* and *in vivo* models [26, 27]. The role of FGF2 in cancer angiogenesis has been assumed based on its production by most cancer cell lines [28, 29]. However, it is important to note that when breast cancer cell lines are evaluated *in vitro* or in xenograft models to investigate tumor-stroma interactions, the results are often not applicable to *in vivo* tumorigenesis due to differences in the composition of the local extracellular matrix[30-33] and the absence of various tissue specific stromal interactions[34-36]. Furthermore, tumor cells lines are derived from cells at very late stages of tumor progression and selected for growth in cell culture over many passages [37]. At the same time, naturally occurring tumors are rare and unpredictable. In order to overcome these difficulties, we used the MMTV-PyVT mouse model. This model has a high frequency of tumor development [5], where the PyVT is under control of the mouse mammary tumor virus long terminal repeat promoter (MMTV-LTR) [6]. This model has been shown to be an accurate representation

of the development of human breast cancer [3, 4]. The studies described here provide the *in vivo* demonstration that loss of FGF2 confers a less malignant phenotype due to reduced vascularization.

Furthermore, FGF2 and its receptors have been shown to be highly expressed in many breast cancers[38] and has been shown to have direct mitogenic effects in the mammary epithelium as well as activation of migration [17]. Studies have demonstrated a close interaction between hypoxia and heparin sulfate proteoglycans that modulate the levels of FGF2 signaling [39]. However, previous studies described FGF2 as an upstream mediator of angiogenesis[40] that effects further inflammatory responses that are necessary to complete the process of neo-vascularization [18]. In light of these data as well as the findings presented here, we propose that the action of FGF2—while important in initiating angiogenesis in response to hypoxia-induced stress common in the tumor microenvironment as well as direct mitogenic effects through inhibition of p53, upregulation of survivin [41], and induction of mdm2 [42]—may be superceded by other inflammatory mediators following sufficient inflammatory signaling when it is lost. This hypothesis may harbor significant implications for future therapeutics that attempt to target the action of FGF2. Rather than solely targeting the action of FGF2 on mammary epithelium, there will need to be a combination of therapeutics that will prevent bypassing of FGF2-induced angiogenesis.

There are a variety of clinical studies that have shown that low serum levels of FGF2 correlate with a more malignant phenotype [13], larger tumor size, later disease stage [14], and worse overall and disease-free survival [13, 14]. In addition, many pre-clinical studies have also shown that overexpression of FGF2 inhibits growth [43], although the exact pathway has yet to be elucidated [44]. In contrast, our study shows that the loss of one or both copies of FGF2 results in smaller tumors. Further studies are needed to reconcile this incongruity.

The primary goal of this study was to analyze the contribution of FGF2 to tumor development and progression to malignancy *in vivo* through gene knockout. Because FGF2 KO mice were shown to have delayed tumor development and growth compared to PyVT controls with endogenous levels of FGF2, we suggest that FGF2 promotes tumor angiogenesis and progression to malignancy. This experiment has validated the concept that FGF2 blockade in the context of breast cancer can be a beneficial therapeutic intervention. The data obtained here demonstrate that the PyVT mammary tumors are sensitive to the absence of FGF2 and identify the PyVT model as an important preclinical model to evaluate therapies aiming to remove FGF2 function. Our results support the conventional view that, presumably because of its powerful mitogenic activity, FGF2 is a pro-tumorigenic growth factor.

LEGENDS

Figure 1. Delayed progression of tumorigenesis and decreased tumor size in knockout and heterozygous mice for FGF2 as compared to normal mice. A: Tumor onset of tumor-bearing PyVT+ FGF2 KO and WT mice. ($P<0.05$) **B:** Tumor onset of tumor-bearing PyVT+ FGF2 het vs. WT mice. ($P<0.05$) **C:** Mammary tumor wet weight in tumor bearing WT and FGF2 KO mice upon harvest at 110-125 days ($P<0.05$).

Figure 2. Ductal morphology in normal (PyVT-) mammary gland is comparable to those in PyVT+ mice. A: Masson's Trichrome staining of mammary gland from normal PyVT- mouse at 10 weeks of age, with collecting ducts (CD) and adipose tissue (AT) indicated. **B:** High magnification of inset from panel A with myoepithelial cells (MEC) and luminal breast epithelial cells (BEC) indicated. **C:** Masson's Trichrome staining of mammary gland prepared from a normal lactating mouse at 26 weeks of age. **D.** High magnification of inset from panel C. **E:** Masson's Trichrome staining of mammary gland from PyVT+ mouse at 22 weeks of age. **F.** High magnification of inset from panel E. **G:** H&E staining of mammary gland from FGF2 KO mouse at 14 weeks of age. **H.** High magnification of inset from panel G. Scale bars: 200 μm (C, E, G); 100 μm (A, D, F, H); 50 μm (B).

Figure 3. Loss of FGF2 results in decreased tumor vascularization. A: Immunolocalization of Factor VIII (arrows) in a normal mouse mammary fat pad at 17 weeks of age. **B.** High magnification of inset from panel A. **C:** Immunolocalization of Factor VIII in an FGF2 KO mouse mammary fat pad at 19 weeks of age. **D.** High magnification of panel C. **E:** Immunolocalization of Factor VIII in a WT mouse mammary fat pad at 22 weeks of age. **F.** High magnification of inset in panel E. **G:** Immunolocalization of Factor VIII in a FGF2 KO mouse mammary fat pad at 14 weeks of age. **H.** High magnification of inset in panel G. MEC, myoepithelial cells. BEC, luminal breast epithelial cells. AT, adipose tissue. Scale bars : 100 μm (A, C, E, G); 500 μm (B, D, F, H). **I:** Quantification of blood vessel density decrease in tumor bearing FGF2 KO (n=3), FGF2 het (n=3) and WT (n=4) mice. The decrease in vascularization between WT and FGF2 KO was statistically significant ($P<0.05$).

Figure 4. FGF2 present in normal (non-PyVT) and WT while absent in FGF2 KO mice. A: Immunolocalization of FGF2 in a normal mouse mammary fat pad at 17 weeks of age. **B:** High magnification of panel A. **C:** Immunolocalization of FGF2 in a WT mouse mammary fat pad. **D:** High magnification of inset in panel C. **E:** Immunolocalization of FGF2 in FGF2 KO mouse mammary tumor at

19 weeks of age. **F.** High magnification of inset in panel E. MEC, myoepithelial cells. BEC, luminal breast epithelial cells. BV, blood vessel. AT, adipose tissue. CD, collecting ducts. Scale bars: 100 µm (A, C, E); 50 µm (B, D, F).

Figure 5. FGF receptor type 1 (FGFR1) present throughout MFPs in WT, and FGF2-KO mice. A: Immunolocalization of FGFR1 in a normal (non-PyVT) mouse mammary tissue at 10 weeks of age. **B**: High magnification of inset from panel A. **C:** Immunolocalization of FGFR1 in a WT mouse mammary tumor at 22 weeks of age. **D:** High magnification of inset from panel C. **E:** Immunolocalization of FGFR1 in a FGF2-KO mouse mammary tumor at 14 weeks of age. **F:** High magnification of inset from panel D. MEC, myoepithelial cells. BEC, luminal breast epithelial cells. BV, blood vessel. AT, adipose tissue. CD, collecting ducts. Scale bars: 100 µm (A, C, E); 50 µm (B, D, F).

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Figure 1

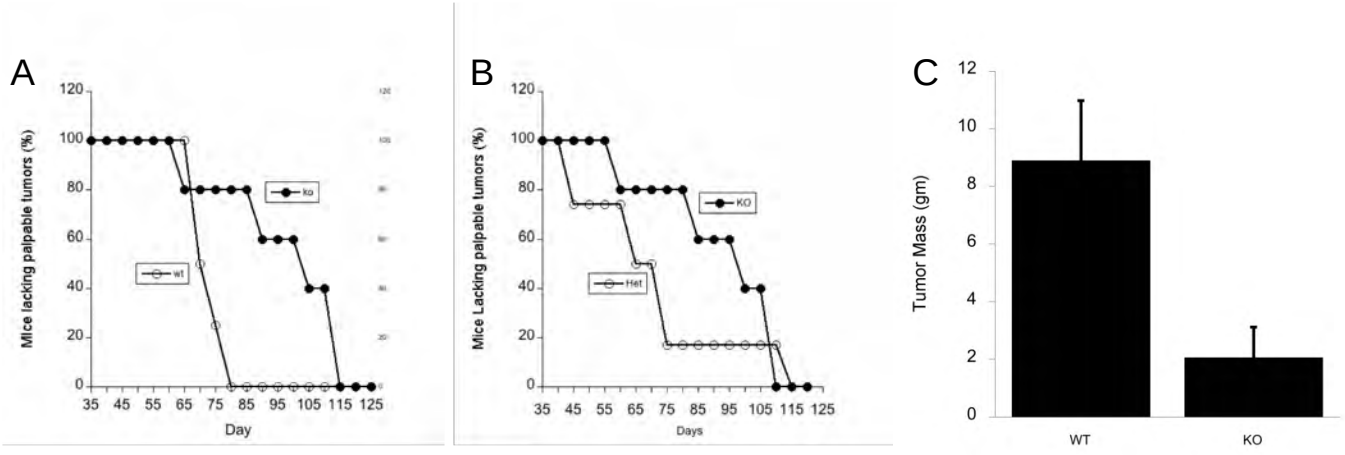
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Figure 2 A-D PyVT- mice

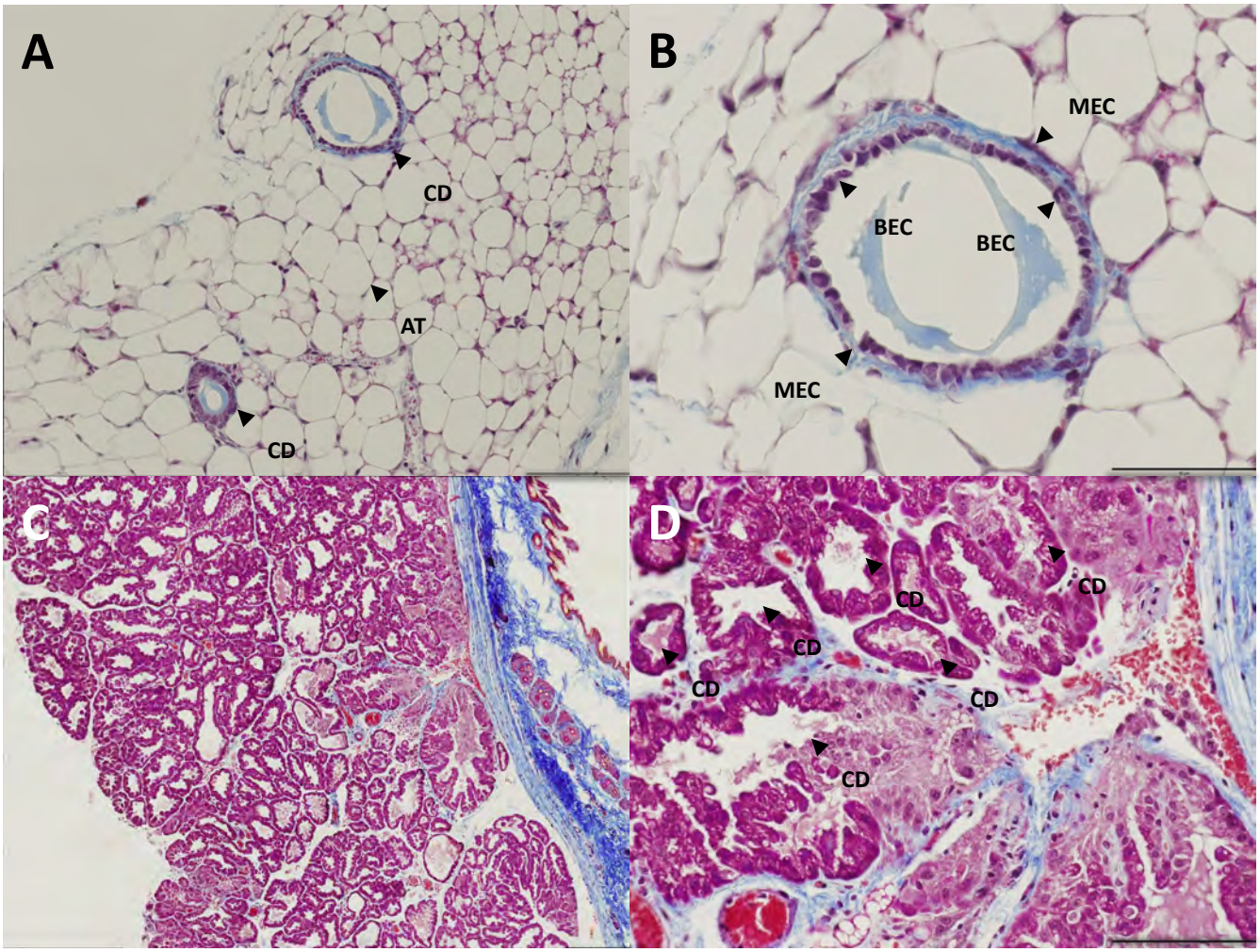

Figure 2 E-H PyVT+ mice

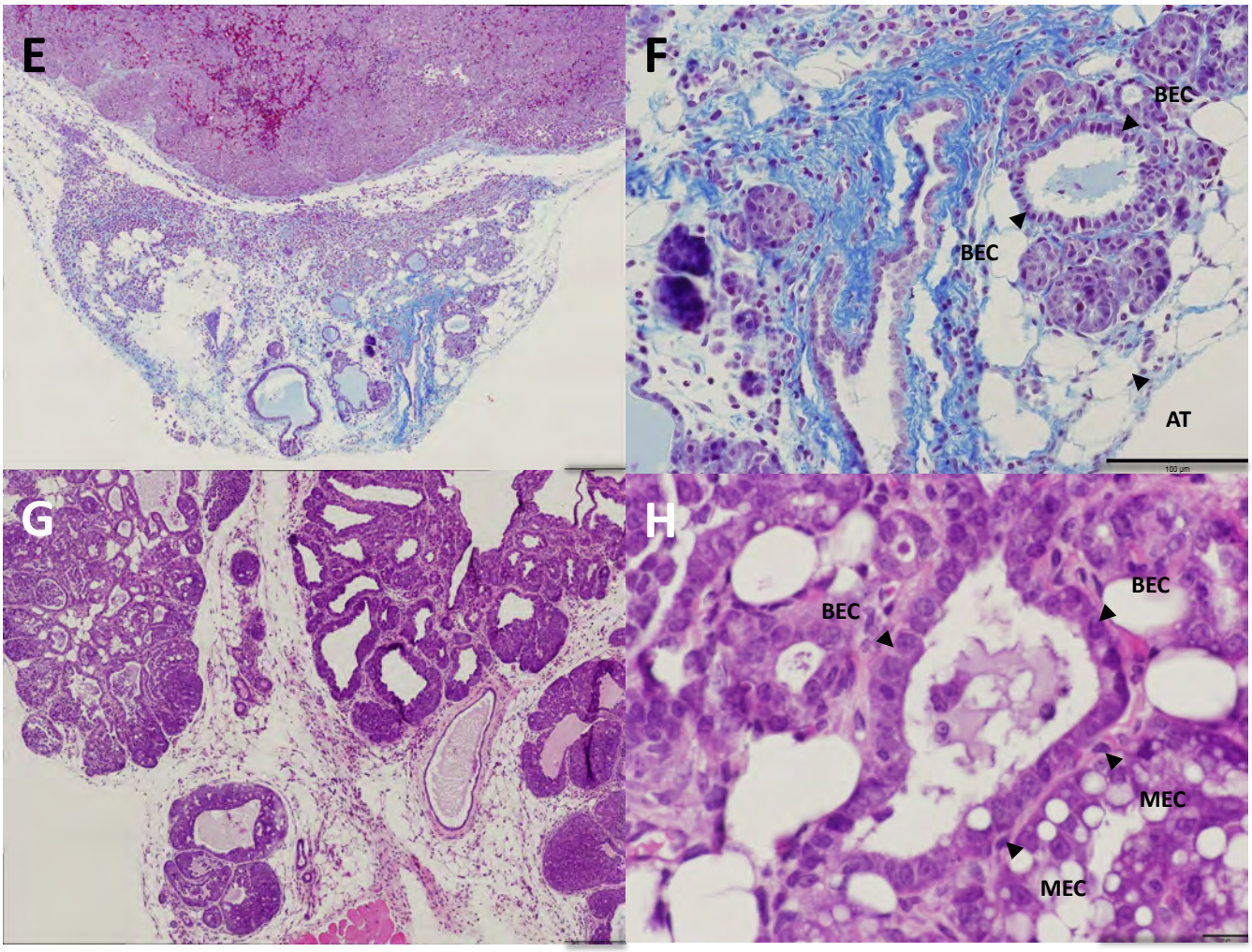

Figure 3 A-D

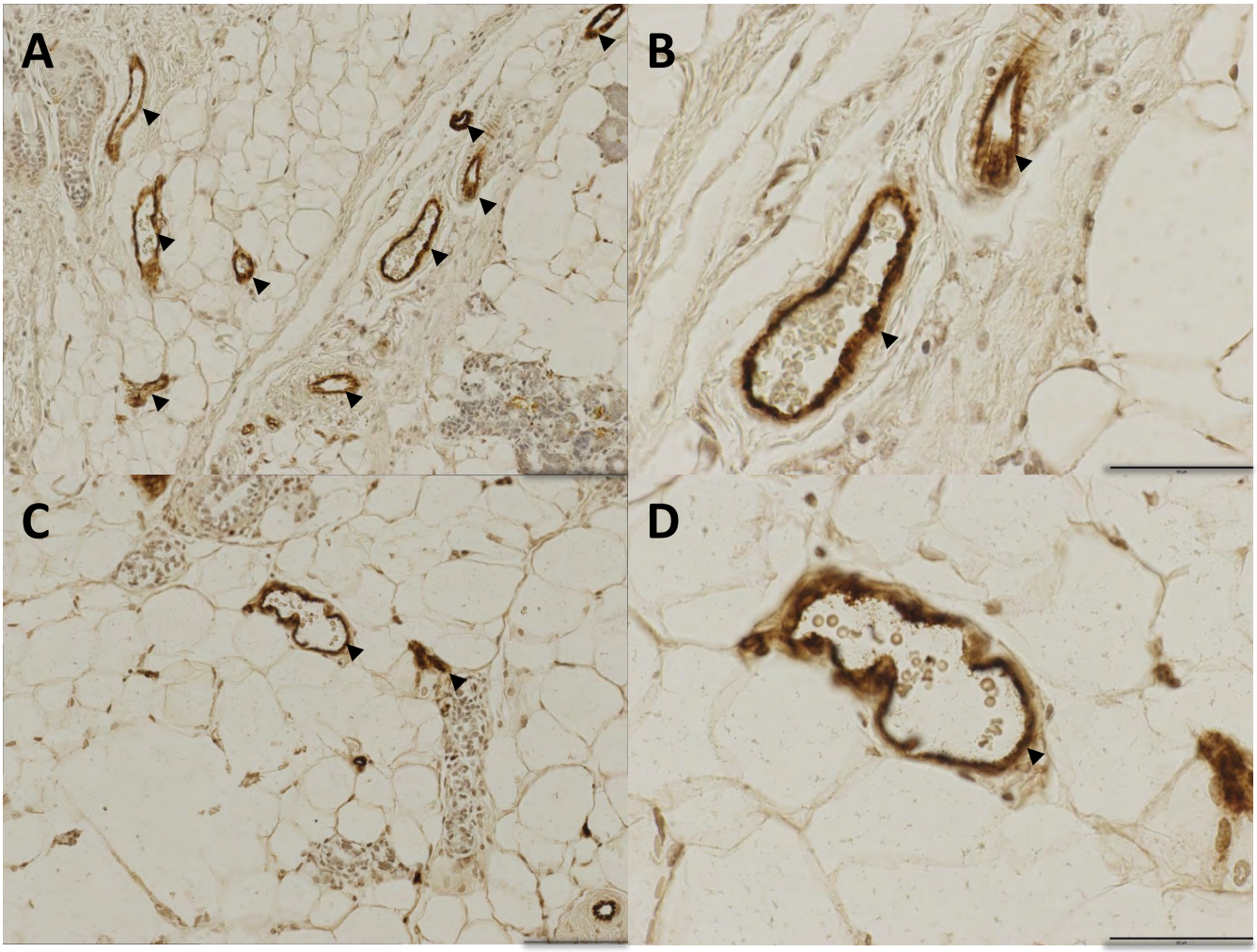

Figure 3 E-H

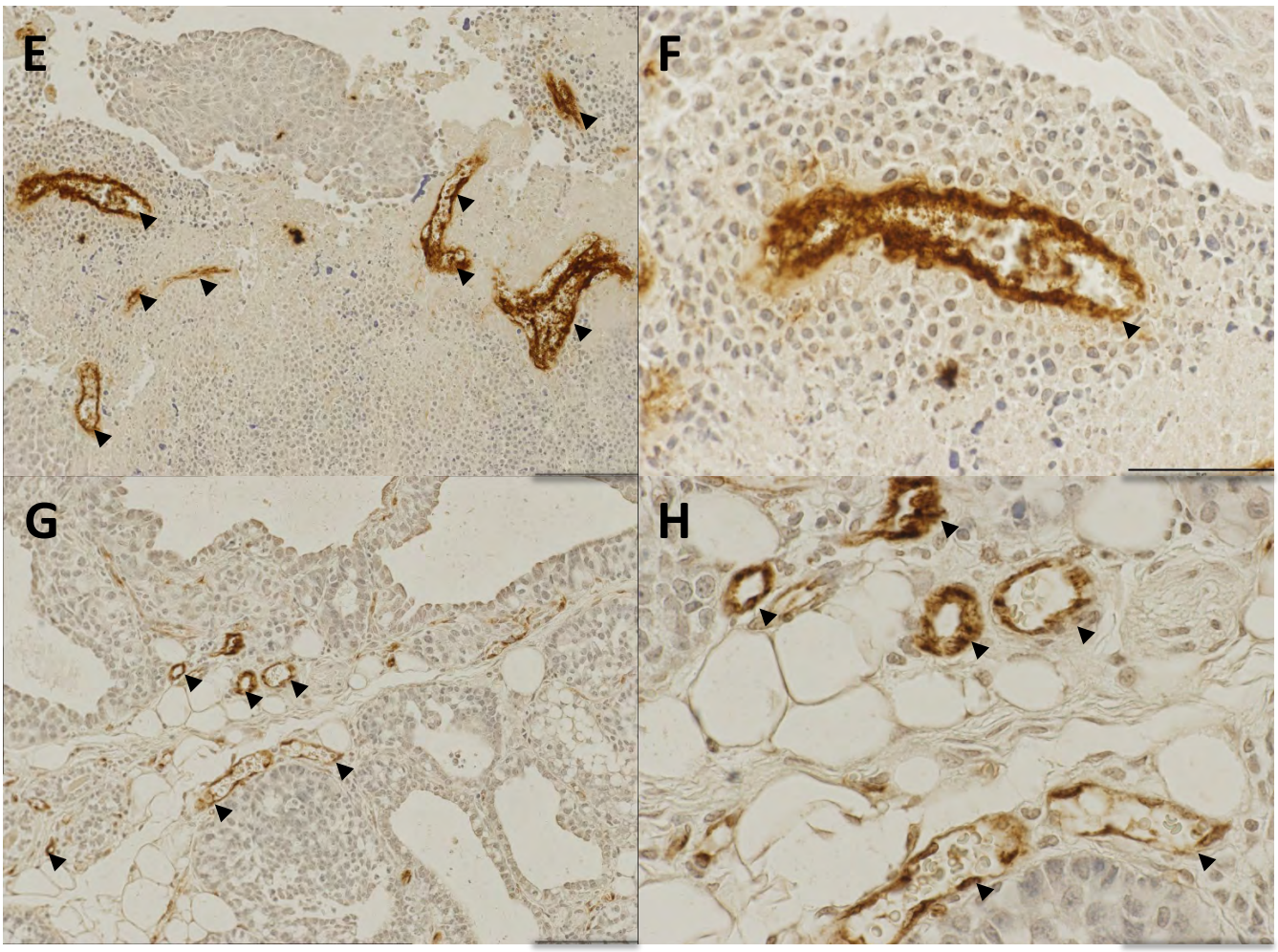

Figure 3 I

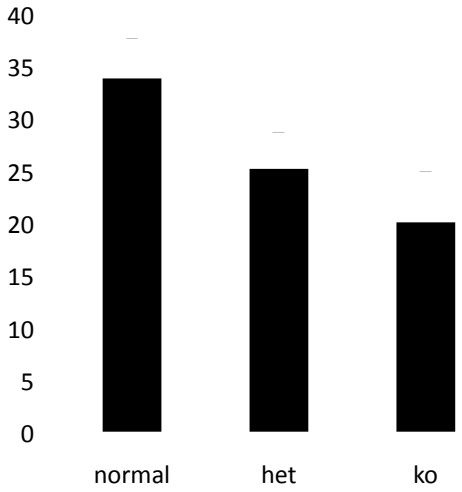

Figure 4 A-F

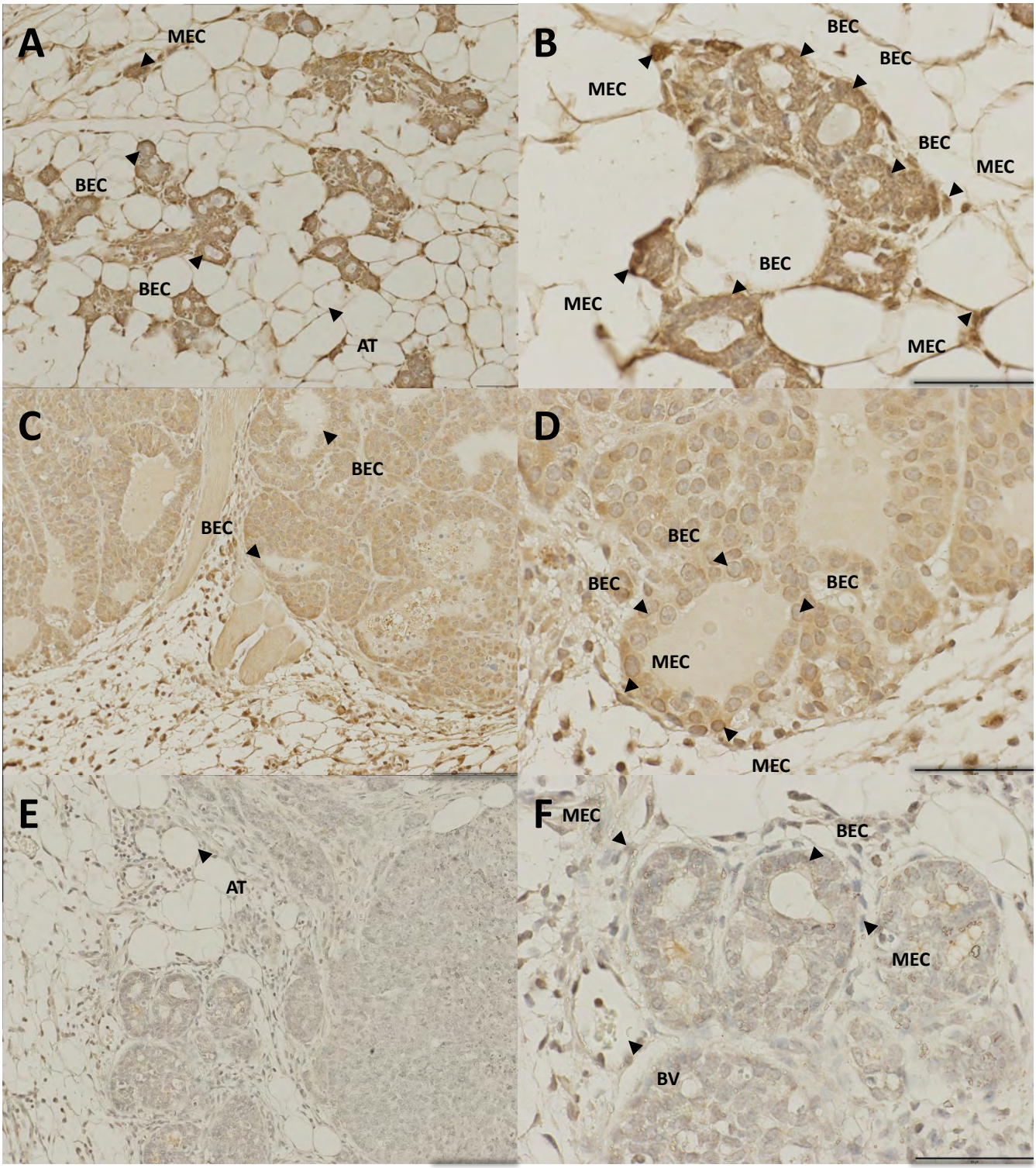
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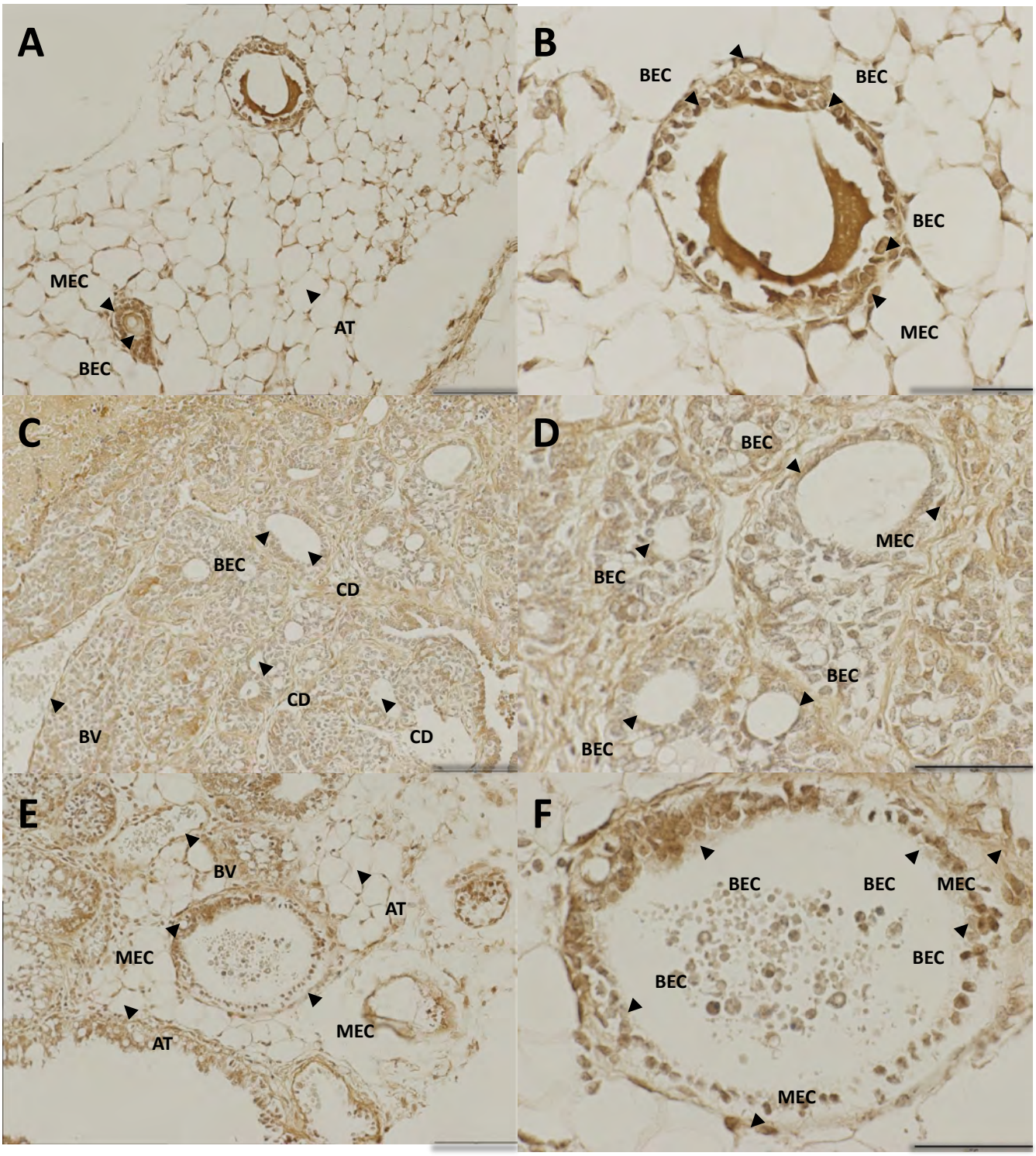
Figure 5 A-F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8. Supporting Data

Figure S1: FGF2 immunohistochemical staining to determine the presence and localization in mammary tumor ductal epithelium. To characterize the role of FGF2 in normal and tumor-bearing mammary tissue, immunohistochemical staining was performed to determine the presence and localization of FGF2 in breast tumor ductal epithelium (also summarized in appendix). Diffuse staining for FGF2 was present throughout the myo- and basal ductal epithelium of normal (non-PyVT+) (Figure 4A,B) and transgenic PyVT mammary tumors (Figure 4C,D) but absent in FGF2 KO (Figure 4E,F) mice. The similar staining pattern seen in normal and transgenic PyVT tumors indicated that the introduction of the PyVT oncogene had a limited effect on the expression and localization of endogenous FGF2. In FGF KO tumors, the diffuse staining pattern described earlier was absent, confirming loss of FGF2 immunoreactivity in the knockout.